

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

B19

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : A61K 48/00		A2	(11) International Publication Number: WO 95/24929 (43) International Publication Date: 21 September 1995 (21.09.95)
<p>(21) International Application Number: PCT/US95/03307</p> <p>(22) International Filing Date: 15 March 1995 (15.03.95)</p> <p>(30) Priority Data: 213,668 15 March 1994 (15.03.94) US</p> <p>(71) Applicant: BROWN UNIVERSITY RESEARCH FOUNDATION [US/US]; Box 1949, Providence, RI 02912 (US).</p> <p>(72) Inventors: MATHIOWITZ, Edith; 184 Rawson Road, Brookline, MA 02146 (US). JONG, Yong, Shik; Hankang Minyoung Apartment L-301, Dongbu-Ichon Dong, Seoul (KR). BOEKELHEIDE, Kim; 154 Pine Hill Road, Wakefield, RI 02879 (US).</p> <p>(74) Agent: PABST, Patrea, L.; Arnall Golden & Gregory, 2800 One Atlantic Center, 1201 West Peachtree Street, Atlanta, GA 30309-3450 (US).</p>		<p>(81) Designated States: AU, CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).</p> <p>Published <i>Without international search report and to be republished upon receipt of that report.</i></p>	
<p>(54) Title: POLYMERIC GENE DELIVERY SYSTEM</p> <p>(57) Abstract</p> <p>A means for obtaining efficient introduction of exogenous genes into a patient, with long term expression of the gene, is disclosed. The gene, under control of an appropriate promoter for expression in a particular cell type, is encapsulated or dispersed with a biocompatible, preferably biodegradable polymeric matrix, where the gene is able to diffuse out of the matrix over an extended period of time, for example, a period of three to twelve months or longer. The matrix is preferably in the form of a microparticle such as a microsphere (where the gene is dispersed throughout a solid polymeric matrix) or microcapsule (gene is stored in the core of a polymeric shell), a film, an implant, or a coating on a device such as a stent. The size and composition of the polymeric device is selected to result in favorable release kinetics in tissue. The size is also selected according to the method of delivery which is to be used, typically injection or administration of a suspension by aerosol into the nasal and/or pulmonary areas. The matrix composition can be selected to not only have favorable degradation rates, but to be formed of a material which is bioadhesive, to further increase the effectiveness of transfer when administered to a mucosal surface.</p>			

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	GB	United Kingdom	MR	Mauritania
AU	Australia	GE	Georgia	MW	Malawi
BB	Barbados	GN	Guinea	NE	Niger
BE	Belgium	GR	Greece	NL	Netherlands
BF	Burkina Faso	HU	Hungary	NO	Norway
BG	Bulgaria	IE	Ireland	NZ	New Zealand
BJ	Benin	IT	Italy	PL	Poland
BR	Brazil	JP	Japan	PT	Portugal
BY	Belarus	KE	Kenya	RO	Romania
CA	Canada	KG	Kyrgyzstan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo	KR	Republic of Korea	SE	Sweden
CH	Switzerland	KZ	Kazakhstan	SI	Slovenia
CI	Côte d'Ivoire	LI	Liechtenstein	SK	Slovakia
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CN	China	LU	Luxembourg	TD	Chad
CS	Czechoslovakia	LV	Latvia	TG	Togo
CZ	Czech Republic	MC	Monaco	TJ	Tajikistan
DE	Germany	MD	Republic of Moldova	TT	Trinidad and Tobago
DK	Denmark	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	US	United States of America
FI	Finland	MN	Mongolia	UZ	Uzbekistan
FR	France			VN	Viet Nam
GA	Gabon				

POLYMERIC GENE DELIVERY SYSTEM

Background of The Invention

The present invention is generally in the area of drug delivery devices and is specifically in the 5 area of polymeric drug delivery devices.

"Gene Therapy" is generally defined as the introduction and expression of an exogenous gene in an animal to supplement or replace a defective or missing gene. Examples that have received a great 10 deal of recent attention include the genes missing in cystic fibrosis and severe combined immunodeficiency. Although tremendous progress has been made in the area of gene therapy, obtaining high level and long term expression of the desired 15 proteins remains elusive.

In the majority of cases, a retroviral vector is used to introduce the gene to be expressed into appropriate cells. Gene transfer is most commonly achieved through a cell-mediated *ex vivo* therapy in 20 which cells from the blood or tissue are genetically modified in the laboratory and subsequently returned to the patient. The clinical studies by Steven Rosenberg, et al., "Immunotherapy of patients with metastatic melanoma using tumor- 25 infiltrating lymphocytes and IL-2", Preliminary report, *New England J. Med.*, 319 (1988) 1676-1680, using *in vitro*-activated LAK and TIL for tumor destruction illustrates this approach. In other cases, the vector carrying the gene to be expressed 30 is introduced into the patient, for example, by inhalation into the lungs in the case of cystic fibrosis. Transfected cells have also been implanted, alone or encapsulated within a protective membrane that protects the cells from 35 the inflammatory response of the body while at the same time allowing the gene product to diffuse out of the membrane. There have also been reports of

the direct injection of an exogenous gene in combination with an appropriate promoter, into tissue, with some transient expression being noted.

Viral vectors have been widely used in gene transfer, due to the relatively high efficiency of transfection and potential long term effect through the actual integration into the host's genome. 5 However, there are still concerns about the risks involved in the use of viruses. Activation of 10 proto-oncogenes and reversion to wild-type viruses from replication incompetent viruses are some important potential hazards of viral delivery of genes.

Since the discovery that naked DNA is taken up 15 by muscle cells and transiently expressed *in vivo*, and subsequent reports, by Wolff, Jon Aal, et, "Direct gene transfer into mouse muscle *in vivo*," *Science*, 247, 1465-1468, 1990; and Wolff, Jon A, "Human dystrophin expression in mdx mice after 20 intramuscular injection of DNA constructs," *Nature*, 352, 815-818, 1991, there has been increasing interest in using non-viral vehicles for *in vivo* transfections.

Plasmid DNA, which can function episomally, 25 has been used with liposome encapsulation, CaPO_4 precipitation and electroporation as an alternative to viral transfections. Recent clinical trials with liposome encapsulated DNA in treating melanoma illustrates this approach to gene therapy, as 30 reported by Nabel, J.G., et al., "Direct gene transfer with DNA-liposome complexes in melanoma: Expression, biological activity and lack of toxicity in humans", *Proc. Nat. Acad. Sci. U.S.A.*, 90 (1993) 11307-11311. A foreign gene coding for 35 HLA-B was introduced into subcutaneous sites of melanoma tumors. Expression of the new gene and the absence of an anti-DNA host response was

confirmed. Wolff, Jon A, "Persistence of plasmid DNA and expression in rat brain cells *in vivo*," *Experimental Neurology*, 115, 400-413, 1992, also reported expression of plasmid DNA. Thus, direct 5 gene transfer offers the potential to introduce DNA encoding proteins to treat human diseases.

The mechanisms for cellular uptake of exogenous DNA and subsequent expression are not clear but gene transfer with naked DNA is 10 associated with several characteristics. Unlike in the case of oligonucleotides, which are typically a maximum of twenty to thirty nucleotides in length, genes encoding most molecules of therapeutic interest are quite large, and therefore 15 considerably more difficult to introduce into cells other than through retroviral vector, or *in vitro*, by chemical manipulation, so that the efficiency of transfer is low. In most reported cases to date, only transient high level expression of up to a few 20 weeks or months has been observed. Although low level expression and short term expression are two important drawbacks with direct DNA transfer, transfections with naked DNA have several 25 advantages over viral transfers. Most importantly, concerns related to the immunogenicity and transforming capability of viruses are avoided. In addition, naked DNA is easy to produce in large quantities, is inexpensive, and can be injected at high concentration into localized tissue sites 30 allowing gene expression *in situ* without extensive *ex vivo* therapy.

The following additional articles review the current state of gene therapy and the problems associated therewith: Blau, Helen M, "Muscling in 35 on gene therapy," *Nature*, 364, 673-675, 1993; Cohen, Jon, "Naked DNA points way to vaccines," *Science*, 259, 1691-1692, 1993; Dagani, Ron, "Gene

therapy advance, anti-HIV antibodies work inside cells," *C&EN*, 3-4, 1993; Felgner, Philip L, "Lipofectamine reagent: A new, higher efficiency polycationic liposome transfection reagent," 5 *Focus/Gibco*, 15, 73-78, 1993; Liu, Margaret A et al., "Heterologous protection against influenza by injection of DNA encoding a viral protein," *Science*, 259, 1745-1749, 1993; Marx, Jean, "A first step toward gene therapy for hemophilia B," 10 *Science*, 262, 29-30, 1993; Mulligan, Richard C, "The basic science of gene therapy," *Science*, 260, 926-931, 1993; Nicolau, Claudeal, et, "In vivo expression of rat insulin after intravenous administration of the liposome-entrapped gene for 15 rat insulin I," *Proc. Natl. Acad. Sci. USA*, 80, 1068-1072, 1983; Partridge, Terence A, "Muscle transfection made easy," *Nature*, 352, 757-758, 1991; Wilson, James M, "Vehicles for gene therapy," *Nature*, 365, 691-692, 1993; Wivel, et al., "Germ- 20 line gene modification and disease prevention: Some medical and ethical perspectives," *Science*, 262, 533-538, 1993; and Woo, Savio L Cal, et, "In vivo gene therapy of hemophilia B: sustained partial correction in Factor IX-deficient dogs," *Science*, 25, 262, 117-119, 1993.

Gene therapy is one of the most promising areas of research today. It would therefore be extremely useful if one had an efficient way to introduce genes into cells which yielded long term 30 expression.

It is therefore an object of the present invention to provide a means for efficient transfer of exogenous genes to cells in a patient.

It is a further object of the present 35 invention to provide a means for long term expression of exogenous genes in patients.

It is a further object of the present invention to provide a means for increasing or decreasing the inflammatory response to implanted polymeric devices.

- 5 It is a still further object of the present invention to provide a method for immunization of individuals over a more prolonged period of time than is achieved by a single or multiple immunization protocol.
- 10 It is another object of the present invention to provide a method for targeting of gene delivery either to tissue cells or to inflammatory type cells.

Summary of the Invention

15 A means for obtaining efficient introduction of exogenous genes into a patient, with long term expression of the gene, is disclosed. The gene, under control of an appropriate promoter for expression in a particular cell type, is

20 encapsulated or dispersed with a biocompatible, preferably biodegradable polymeric matrix, where the gene is able to diffuse out of the matrix over an extended period of time, for example, a period of three to twelve months or longer. The matrix is

25 preferably in the form of a microparticle such as a microsphere (where the gene is dispersed throughout a solid polymeric matrix) or microcapsule (gene is stored in the core of a polymeric shell), although other forms including films, coatings, gels,

30 implants, and stents can also be used. The gene, or other DNA or RNA to be delivered, can be incorporated directly into the polymer, or first incorporated into another material enhancing penetration of the DNA through the cell wall, such

35 as liposomes or surfactants. The size and composition of the polymeric device is selected to

result in favorable release kinetics in tissue. The size is also selected according to the method of delivery which is to be used, typically injection into a tissue or administration of a 5 suspension by aerosol into the nasal and/or pulmonary areas. The matrix composition can be selected to not only have favorable degradation rates, but to be formed of a material which is bioadhesive, to further increase the effectiveness 10 of transfer when administered to a mucosal surface, or selected not to degrade but to release by diffusion over an extended period.

Examples demonstrate the effectiveness of the system in animals.

15 **Detailed Description of the Invention**

Gene transfer is achieved using a polymeric delivery system which releases entrapped genes, usually in combination with an appropriate promoter for expression of the gene, into surrounding 20 tissue. Efficacy of transfer is achieved by: a) releasing the gene for prolonged period of time, b) minimizing diffusion of the gene out of the delivery system (due to its size) so that release is predominantly degradation dependent, and c) 25 improving the transient time of expression and the low infection seen by direct gene therapy. In case of non-erodible polymers, the device is formulated so that the gene is released via diffusion. This is achieved by creating porous systems or adding 30 soluble bulking agents that create pores as they leach out of the system.

The Polymeric Matrices

Selection of Polymer

Both non-biodegradable and biodegradable 35 matrices can be used for delivery of genes, although biodegradable matrices are preferred.

- These may be natural or synthetic polymers, although synthetic polymers are preferred due to the better characterization of degradation and release profiles. The polymer is selected based on
- 5 the period over which release is desired, generally in the range of at least three months to twelve months, although longer periods may be desirable. In some cases linear release may be most useful, although in others a pulse release or "bulk
- 10 release" may provided more effective results. The polymer may be in the form of a hydrogel (typically in absorbing up to about 90% by weight of water), and can optionally be crosslinked with multivalent ions or polymers.
- 15 High molecular weight genes can be delivered partially by diffusion but mainly by degradation of the polymeric system. In this case, biodegradable polymers, bioerodible hydrogels, and protein delivery systems are particularly preferred.
- 20 Representative synthetic polymers are: polyamides, polycarbonates, polyalkylenes, polyalkylene glycols, polyalkylene oxides, polyalkylene terephthalates, polyvinyl alcohols, polyvinyl ethers, polyvinyl esters, polyvinyl halides,
- 25 polyvinylpyrrolidone, polyglycolides, polysiloxanes, polyurethanes and co-polymers thereof, alkyl cellulose, hydroxyalkyl celluloses, cellulose ethers, cellulose esters, nitro celluloses, polymers of acrylic and methacrylic
- 30 esters, methyl cellulose, ethyl cellulose, hydroxypropyl cellulose, hydroxy-propyl methyl cellulose, hydroxybutyl methyl cellulose, cellulose acetate, cellulose propionate, cellulose acetate butyrate, cellulose acetate phthalate,
- 35 carboxylethyl cellulose, cellulose triacetate, cellulose sulphate sodium salt, poly(methyl methacrylate), poly(ethyl methacrylate),

poly(butylmethacrylate), poly(isobutyl methacrylate), poly(hexylmethacrylate), poly(isodecyl methacrylate), poly(lauryl methacrylate), poly(phenyl methacrylate),
5 poly(methyl acrylate), poly(isopropyl acrylate), poly(isobutyl acrylate), poly(octadecyl acrylate), polyethylene, polypropylene, poly(ethylene glycol), poly(ethylene oxide), poly(ethylene terephthalate), poly(vinyl alcohols), poly(vinyl acetate, poly
10 vinyl chloride, polystyrene and polyvinylpyrrolidone.

Examples of non-biodegradable polymers include ethylene vinyl acetate, poly(meth)acrylic acid, polyamides, copolymers and mixtures thereof.

15 Examples of biodegradable polymers include synthetic polymers such as polymers of lactic acid and glycolic acid, polyanhydrides, poly(ortho)esters, polyurethanes, poly(butic acid), poly(valeric acid), and poly(lactide-co-
20 caprolactone), and natural polymers such as alginic acid and other polysaccharides including dextran and cellulose, collagen, chemical derivatives thereof (substitutions, additions of chemical groups, for example, alkyl, alkylene, hydroxylations, oxidations, and other modifications routinely made by those skilled in the art),
25 albumin and other hydrophilic proteins, zein and other prolamines and hydrophobic proteins, copolymers and mixtures thereof. In general, these materials degrade either by enzymatic hydrolysis or exposure to water *in vivo*, by surface or bulk erosion.

30 Bioadhesive polymers of particular interest include bioerodible hydrogels described by H.S. Sawhney, C.P. Pathak and J.A. Hubell in Macromolecules, 1993, 26, 581-587, the teachings of which are incorporated herein, polyhyaluronic

acids, casein, gelatin, glutin, polyanhydrides, polyacrylic acid, alginate, chitosan, poly(methyl methacrylates), poly(ethyl methacrylates), poly(butylmethacrylate), poly(isobutyl methacrylate), poly(hexylmethacrylate), poly(isodecyl methacrylate), poly(lauryl methacrylate), poly(phenyl methacrylate), poly(methyl acrylate), poly(isopropyl acrylate), poly(isobutyl acrylate), and poly(octadecyl acrylate).

Selection of Matrix Form and Size

In the preferred embodiment, the polymeric matrix is a microparticle between nanometers and one millimeter in diameter, more preferably between 15 0.5 and 100 microns for administration via injection or inhalation (aerosol).

The microparticles can be microspheres, where gene is dispersed within a solid polymeric matrix, or microcapsules, where the core is of a different 20 material than the polymeric shell, and the gene is dispersed or suspended in the core, which may be liquid or solid in nature. Unless specifically defined herein, microparticles, microspheres, and microcapsules are used interchangeably.

25 Alternatively, the polymer may be cast as a thin slab or film, ranging from nanometers to four centimeters, a powder produced by grinding or other standard techniques, or even a gel such as a hydrogel. The polymer can also be in the form of a 30 coating or part of a stent or catheter, vascular graft, or other prosthetic device.

Methods for Making the Matrix

The matrices can be formed by solvent evaporation, spray drying, solvent extraction and 35 other methods known to those skilled in the art.

Microsphere Preparation

Bioerodible microspheres can be prepared using any of the methods developed for making microspheres for drug delivery, for example, as 5 described by Mathiowitz and Langer, J. Controlled Release 5, 13-22 (1987); Mathiowitz, et al., Reactive Polymers 6, 275-283 (1987); and Mathiowitz, et al., J. Appl. Polymer Sci. 35, 755-774 (1988), the teachings of which are incorporated 10 herein. The selection of the method depends on the polymer selection, the size, external morphology, and crystallinity that is desired, as described, for example, by Mathiowitz, et al., Scanning Microscopy 4, 329-340 (1990); Mathiowitz, et al., J. Appl. Polymer Sci. 45, 125-134 (1992); and Benita, 15 et al., J. Pharm. Sci. 73, 1721-1724 (1984), the teachings of which are incorporated herein.

In solvent evaporation, described for example, in Mathiowitz, et al., (1990), Benita, and U.S. 20 Patent No. 4,272,398 to Jaffe, the polymer is dissolved in a volatile organic solvent. The DNA, either in soluble form or dispersed as fine particles, is added to the polymer solution, and the mixture is suspended in an aqueous phase that 25 contains a surface active agent such as poly(vinyl alcohol). The resulting emulsion is stirred until most of the organic solvent evaporates, leaving solid microspheres.

In general, the polymer can be dissolved in 30 methylene chloride. Several different polymer concentrations can be used, for example, between 0.05 and 0.20 g/ml. After loading the solution with DNA, the solution is suspended in 200 ml of vigorously stirring distilled water containing 1% 35 (w/v) poly(vinyl alcohol) (Sigma Chemical Co., St. Louis, MO). After four hours of stirring, the organic solvent will have evaporated from the

polymer, and the resulting microspheres will be washed with water and dried overnight in a lyophilizer.

Microspheres with different sizes (1-1000 microns) and morphologies can be obtained by this method which is useful for relatively stable polymers such as polyesters and polystyrene. However, labile polymers such as polyanhydrides may degrade due to exposure to water. For these polymers, hot melt encapsulation and solvent removal may be preferred.

In hot melt encapsulation, the polymer is first melted and then mixed with the solid particles of DNA, preferably sieved to less than 50 μm . The mixture is suspended in a non-miscible solvent such as silicon oil and, with continuous stirring, heated to 5°C above the melting point of the polymer. Once the emulsion is stabilized, it is cooled until the polymer particles solidify.

The resulting microspheres are washed by decantation with petroleum ether to give a free-flowing powder. Microspheres with diameters between one and 1000 microns can be obtained with this method. The external surface of spheres prepared with this technique are usually smooth and dense. This procedure is useful with water labile polymers, but is limited to use with polymers with molecular weights between 1000 and 50000.

Solvent removal was primarily designed for use with polyanhydrides. In this method, the drug is dispersed or dissolved in a solution of a selected polymer in a volatile organic solvent like methylene chloride. The mixture is then suspended in oil, such as silicon oil, by stirring, to form an emulsion. Within 24 hours, the solvent diffuses into the oil phase and the emulsion droplets harden into solid polymer microspheres. Unlike solvent

evaporation, this method can be used to make microspheres from polymers with high melting points and a wide range of molecular weights.

Microspheres having a diameter between one and 300 microns can be obtained with this procedure. The external morphology of the spheres is highly dependent on the type of polymer used.

In spray drying, the polymer is dissolved in methylene chloride (0.04 g/ml). A known amount of active drug is suspended (if insoluble) or co-dissolved (if soluble) in the polymer solution.

The solution or the dispersion is then spray-dried. Typical process parameters for a mini-spray drier are as follows: polymer concentration = 0.04 g/ml, inlet temperature = 24°C, outlet temperature = 13 to 15°C, aspirator setting = 15, pump setting = 10 ml/min, spray flow = 600 NLh⁻¹, and nozzle diameter = 0.5 mm. Microspheres ranging in diameter between one and ten microns can be obtained with a morphology which depends on the selection of polymer.

Double walled microspheres can be prepared according to U.S. Patent No. 4,861,627 to Mathiowitz.

Hydrogel microspheres made of gel-type polymers such as alginate or polyphosphazenes or other dicarboxylic polymers can be prepared by dissolving the polymer in an aqueous solution, suspending the material to be incorporated into the mixture, and extruding the polymer mixture through a microdroplet forming device, equipped with a nitrogen gas jet. The resulting microspheres fall into a slowly stirring, ionic hardening bath, as described, for example, by Salib, et al., Pharmazeutische Industrie 40-11A, 1230 (1978), the teachings of which are incorporated herein. The advantage of this system is the ability to further

- modify the surface of the microspheres by coating them with polycationic polymers such as polylysine, after fabrication, for example, as described by Lim, et al., J. Pharm. Sci. 70, 351-354 (1981).
- 5 For example, in the case of alginate, a hydrogel can be formed by ionically crosslinking the alginate with calcium ions, then crosslinking the outer surface of the microparticle with a polycation such as polylysine, after fabrication.
- 10 The microsphere particle size will be controlled using various size extruders, polymer flow rates and gas flow rates.

Chitosan microspheres can be prepared by dissolving the polymer in acidic solution and 15 crosslinking with tripolyphosphate. For example, carboxymethylcellulose (CMC) microsphere are prepared by dissolving the polymer in an acid solution and precipitating the microspheres with lead ions. Alginate/polyethylene imide (PEI) can 20 be prepared to reduce the amount of carboxyl groups on the alginate microcapsules. Table 1 summarizes various hydrogels, concentrations, ionic baths, and stirring rates used to manufacture them.

Table 1: Preparation of Hydrogel Matrices

	Hydrogel	Hydrogel dissolving bath	ionic bath	stirring	concen.	pH	Temp °C	concen. (w/v)	rate (rpm)
	chitosan	1.0%	5.0	23	3%	tripoly- phosphate		170	
30	alginate	2.0%	7.4	50	1.3%	calcium chloride		160	
	alginate/ PEI	2.0%/ 6.0%	7.4	50	1.3%	calcium chloride		160	
35	Carboxy methyl cellulose	2.0%	7.4	50	10.0%	lead nitrate		100	

Other device forms

Other delivery systems including films, coatings, pellets, slabs, and devices can be fabricated using solvent or melt casting, and 40 extrusion, as well as standard methods for making composites. The polymer can be produced by first

mixing monomers and DNA as described by Sawhney, et al., and polymerizing the monomers with UV light. The polymerization can be carried out *in vitro* as well as *in vivo*. Thus, any biocompatible glue

5 could be also used to incorporate the DNA.

Loading of Gene

The range of loading of the gene to be delivered is typically between about 0.01% and 90%, depending on the form and size of the gene to be

10 delivered and the target tissue.

Selection of Genes to be Incorporated

Any genes that would be useful in replacing or supplementing a desired function, or achieving a desired effect such as the inhibition of tumor

15 growth, could be introduced using the matrices described herein. As used herein, a "gene" is an isolated nucleic acid molecule of greater than thirty nucleotides, preferably one hundred nucleotides or more, in length.

20 Examples of genes which replace or supplement function include the genes encoding missing enzymes such as adenosine deaminase (ADA) which has been used in clinical trials to treat ADA deficiency and cofactors such as insulin and coagulation factor

25 VIII.

Genes which effect regulation can also be administered, alone or in combination with a gene supplementing or replacing a specific function. For example, a gene encoding a protein which

30 suppresses expression of a particular protein-encoding gene, or *vice versa*, which induces expression of a protein-encoding gene, can be administered in the matrix.

35 Examples of genes which are useful in stimulation of the immune response include viral antigens and tumor antigens, as well as cytokines

(tumor necrosis factor) and inducers of cytokines (endotoxin), and various pharmacological agents.

The chronic immune response to the polymeric matrix is mediated by the action of a variety of 5 growth factors including epidermal growth factor (EGF), platelet-derived growth factor (PDGF), fibroblast growth factors (FGFs), transforming growth factors (TGF- α and TGF- β , interleukin-1 (IL-1), and tumor necrosis factor (TNF). Inhibitors of 10 these inflammatory mediators in combination with a gene to be delivered other than the immune inhibitor would be effective in decreasing the normal inflammatory response directed toward the polymeric matrix. By inhibiting the amount of 15 encapsulation of the matrix, the effective release would be further extended. Examples of materials which could inhibit encapsulation include antisense mRNA to suppress fibrin or collagen formation, inhibitors of EGF, PDGF, FGFs, TGF- α , TGF- β , IL-1 20 and TNF and anti-inflammatory agents such as corticosteroids and cyclosporin.

Genes can be obtained using literature references or from commercial suppliers. They can be synthesized using solid phase synthesis if 25 relatively small, or obtained in expression vectors, for example, as deposited with the American Type Culture Collection, Rockville, MD.

Selection of vectors to be introduced in combination with the gene.

30 As used herein, vectors are agents that transport the gene into the cell without degradation and include a promoter yielding expression of the gene in the cells into which it is delivered. Promoters can be general promoters, 35 yielding expression in a variety of mammalian cells, or cell specific, or even nuclear versus cytoplasmic specific. These are known to those skilled in the art and can be constructed using

standard molecular biology protocols. Although as demonstrated by the examples, the genes will diffuse out of the polymeric matrix into the surrounding cells where they are expressed, in a preferred embodiment, the genes are delivered in combination with a vector to further enhance uptake and expression. Vectors are divided into two classes:

- 5 a) Biological agents derived from viral, bacterial or other sources.
- 10 b) Chemical/ physical methods that increase the potential for gene uptake, directly introduce the gene into the nucleus or target the gene to a cell receptor.

15 Biological Vectors

Viral vectors have higher transaction (ability to introduce genes) abilities than do most chemical or physical methods to introduce genes into cells.

20 Retroviral vectors are the vectors most commonly used in clinical trials, since they carry a larger genetic payload than other viral vectors. However, they are not useful in non-proliferating cells.

25 Adenovirus vectors are relatively stable and easy to work with, have high titers, and can be delivered in aerosol formulation. However, many people may have pre-existing antibodies negating effectiveness and they are difficult to produce in quantity.

30 Pox viral vectors are large and have several sites for inserting genes, they are thermostable and can be stored at room temperature. However, they cannot be transmitted from host to host and there are some safety issues since they can enter other cells.

35 Plasmids are not integrated into the genome and the vast majority of them are present only from

a few weeks to several months, so they are typically very safe. However, they have lower expression levels than retroviruses and since cells have the ability to identify and eventually shut 5 down foreign gene expression, the continuous release of DNA from the polymer to the target cells substantially increases the duration of functional expression while maintaining the benefit of the safety associated with non-viral transfections.

10 Chemical/physical vectors

Other methods to directly introduce genes into cells or exploit receptors on the surface of cells include the use of liposomes and lipids, ligands for specific cell surface receptors, cell 15 receptors, and calcium phosphate and other chemical mediators, microinjections directly to single cells, electroporation and homologous recombination. The chemical/physical methods have a number of problems, however, and will typically not 20 be used with the polymeric matrices described herein. For example, chemicals mediators are impractical for *in vivo* use: when calcium phosphate is used there appears to be very low transduction rate, when sodium butyrate is used the inserted 25 gene is highly unstable and when glycerol is used inserted gene is rapidly lost.

As demonstrated by the examples, it has been discovered that it is possible to incorporate 30 nucleic acid molecules into liposomes or complexed to liposomes which are then entrapped or otherwise incorporated into the polymeric matrix for delivery to cells. The ratio of liposome to polymer solution is important in determining whether the liposomes will remain as separate entities during 35 the process for incorporation into the polymeric matrix. If the ratio of solvent is too high, the phospholipid will dissolve into the polymer

solvent, rather than remaining as part of the liposome bilayer. This is a function of the liposome composition, polymer concentration, and solvent composition. The liposomes increase the 5 efficiency of the transfer of the DNA into the cells. Liposomes are commercially available from Gibco BRL, for example, as LIPOFECTIN® and LIPOFECTACE®, which are formed of cationic lipids such as N-[1-(2,3 dioleyloxy)-propyl]-n,n,n- 10 trimethylammonium chloride (DOTMA) and dimethyl dioctadecylammonium bromide (DDAB). Numerous methods are also published for making liposomes, known to those skilled in the art.

As demonstrated by the following example, any 15 nucleic acid molecule, including genes as defined above, antisense, ribozymes and ribozyme targeting molecules, and probes, defined as an oligonucleotide of at least fourteen to seventeen nucleotides in length, can be mixed with, or 20 incorporated into, the liposomes. The liposome-nucleic acid molecule mixture is then mixed with a polymer solution. As noted above, the polymer solution can be formed by melting of a polymer, dissolution of a polymer in a solvent, or selection 25 of a polymer that is a liquid under certain conditions, such as an acidic pH or room temperature, which is then solidified by a change in conditions.

Pharmaceutical Compositions

30 When the matrix is in the form of microparticles, the microparticles can be suspended in any appropriate pharmaceutical carrier, such as saline, for administration to a patient. In the most preferred embodiment, the microparticles will 35 be stored in dry or lyophilized form until immediately before administration. They will then

be suspended in sufficient solution for administration.

In some cases, it may be desirable to administer the microparticles in combination with 5 an adjuvant to enhance the inflammatory response against the polymer and thereby increase the likelihood of phagocytosis by macrophages and other hematopoietic cells, with subsequent expression of the gene specifically within these cells, or, in 10 the case where the microparticles contain an anti-cancer agent, to enhance the inflammatory reaction against the tumor cells in combination with the effect of the anti-cancer agent.

The polymeric microparticles can be 15 administered by injection, infusion, implantation, orally (not preferred), or administration to a mucosal surface, for example, the nasal-pharyngeal region and/or lungs using an aerosol, or in a cream, ointment, spray, or other topical carrier, 20 for example, to rectal or vaginal areas.

Other embodiments, such as slabs, coatings, rods, and other devices are preferably administered by implantation in the area where release is desired.

25 The materials can also be incorporated into an appropriate vehicle for transdermal delivery as well as stents. Appropriate vehicles include ointments, lotions, patches, and other standard delivery means.

30 Targeting of cell populations through polymer material characteristics.

Studies with plasmid release using PLA/PCL biodegradable polymers indicate that the majority of transfected cells, assessed with the β -galactosidase reporter gene, are inflammatory cells 35 involved in the "foreign body" response. In general, degrading non-degrading polymers evoke a stronger inflammatory response when compared to

non-biodegrading polymers. A strong foreign body response results in a thick layer of macrophages, fibroblasts, and lymphocytes around the implant. Because the polymer release device relies on diffusion for movement of its particles, a strong inflammatory response will limit the effective distance of diffusion. Accordingly, biodegrading polymers can be used to target inflammatory cells due to the inability of the plasmid DNA (pDNA) to migrate across the reactive tissue layer to the site specific tissue. A more biocompatible material which induces a weaker response from the host will result in a thinner layer of inflammatory cells, enabling the released pDNA to migrate across the inflammatory cells to the indigenous cells to be transfected.

Protection of Plasmid DNA from Immediate Degradation

Tris-EDTA (TE) is the buffer of choice for DNA due primarily to it's inhibitory effect on nucleases. It is known that storage of DNA in other buffers or plain water results in degradation of the DNA even at 4° C (Wolff, et al., *Biotechniques* 11(4), 474-485 (1991)). The polymer release system can be loaded to deliver plain DNA or DNA with buffer salts to be used as carriers or protecting agents depending on the choice of buffer used.

30 Incorporation of Antiinflammatories and Immune Enhancers; Treatment of Cancers

In recent years, considerable attention has been focused on the use of gene therapy to treat various diseases including cancer. Generally, gene therapy for cancer therapeutics either targets the cells of the immune system to enhance their ability to kill malignant cells or directly targets the cancer cells to regulate their proliferation or

enhance some cellular function which will result in a stronger activation of the immune response.

Most types of cancer are characterized by frequent relapses during the course of treatment and the continued non-specific and/or specific activation of the immune system resulting from gene therapy is crucial. Second, cell targeting is a major limitation of current vectors and implantation of a controlled release device directly inside a tumor where the DNA is released locally is one alternative to *ex vivo* therapy or the development of effective ligand specific vectors. As indicated by the prevalence of *ex vivo* therapy, targeting hematopoietic cells is especially difficult. The histological results from the implant site in the studies described in the examples below, reveal a substantial inflammatory response surrounding the intramuscular implant. The well known "foreign body" host response can be used to an advantage as this migration of lymphocytes and antigen presenting cells raises the possibility of directing the transfection to these specific cell populations.

Tumors elicit both the humoral and cell-mediated immune response, and lymphocytes, particularly cytotoxic T cells and NK cells, as well as macrophages, are known to play a crucial role in tumor elimination. Gene therapy for cancer treatment either targets these cells or the malignant cells themselves. An implant releasing naked DNA for long term functional gene transfer which can target inflammatory cells and/or tumor cells could significantly improve cancer therapy.

The approaches used include upregulation of class I MHC expression, transduction of antigen presenting cells with tumor-specific antigens,

cytokine immunotherapy, transfection of tumor cells with tumor suppressor genes and anti-sense therapy.

The malignant transformation of cells is often characterized by a reduction of class I MHC

- 5 expression leading to a severe depression of the CTL-mediated immune response. An increase in class I MHC expression on tumor cells could facilitate the activation of the immune system against these altered self-cells. Transfection of genes for
- 10 cytokines such as tumor necrosis factor (TNF) into tumor cells or tumor suppressor genes such as p53 can be used to limit the ability of tumor cells to multiply. Anti-sense therapy targets cell proliferation or the production of necessary
- 15 proteins such as tumor angiogenesis factor (TAF) by complementary RNA hybridization to block transcription of specific genes.

- The immune system can be activated and induced to attack specific cells using cytokines such as
- 20 Proleukin or monoclonal antibodies. For example, cancer cells proliferate in part due to a decreased immune response against the transformed cells. The matrices described herein provide a means to allow recognition and provocation of a response to cancer
 - 25 cells. For example, genes coding for antigens, such as the aberrant epithelial mucin of breast cancer, and monoclonal antibodies directed against tumor antigens have been shown to have potential in stimulating immune destruction of malignant cells.
 - 30 These genes, alone or in combination with monoclonal antibodies, can be delivered to the tumor sites in the polymeric matrices to achieve inhibition of the tumor cells.

- 35 . Cancer cells can also be treated by introducing chemotherapy drug resistant genes into healthy cells to protect them against the toxicity of drug therapy, or by the insertion of appropriate

vectors containing cytotoxic genes or blocking genes into a tumor mass to eliminate cancer cells. In a preferred embodiment, the immune system is specifically stimulated against antigens or 5 proteins on the surface of the cancer cells.

These approaches can be used *in vitro* and *in vivo*. *In vitro*, the cells can be removed from a patient, the gene inserted into the cell and the cells reintroduced into the patient. *In vivo*, the 10 gene can be directly introduced into the body either systematically or in localized sites.

Another approach is to use suicide genes that cause cell death when they are activated or when their product is combined with a pharmaceutical. 15 The primary limitation of the method is the fact that the gene should be targeted to the cancer cell and not to normal cell. Current approach to overcome the problem is direct injection of the vectors into a localized area where normal cells do 20 not proliferate. This would be greatly facilitated using the polymeric devices described herein. The advantages of polymeric devices in this setting include continuous and protracted release of the incorporated pharmaceutical. This increases the 25 likelihood that the intended purposes, for example, treatment of cancerous cells, will be achieved.

Examples

The method and materials of the present invention will be further understood by reference 30 to the following non-limiting examples.

Example 1: Expression of linear and supercoiled plasmid DNA encapsulated in polymeric implants in muscle tissue of rats.

35 The study described in this example confirms the feasibility of *in vivo* transfections using biodegradable polyester blends to release linear or supercoiled plasmid DNA. Although only short term

expression was studied in this study, polymer devices releasing drugs offer the potential for sustained long term delivery of naked DNA.

Marker genes are used to study the movement of
5 engineered cells containing exogenous genes, as
well as the vectors and genes introduced with the
vectors, to insure that the genes remain where they
are introduced. Almost all of the initial research
into gene therapy is with marker genes. Preferred
10 marker genes are those whose product is innocuous
and which can be readily detected by simple
laboratory tools. An appropriate marker gene is β -
galactosidase (β -gal), since expression is readily
detected by addition of X-gal, a substrate which
15 yields a blue color when the active enzyme is
present.

Encapsulation of linear and supercoiled β -gal coding DNA in a PLA blend

1 g PLA (300K) and 2 g PLA (2K) was dissolved
20 in 10 ml of methylene chloride and 5 drops of Span
85. The mixture was divided into two aliquots of 5
ml and 100 μ l of either circular or linear DNA
(between 1 and 2 mg/ml diluted 1:5 in buffer) was
introduced into the aliquots. Each mixture was
25 mixed well and aliquoted into glass vials (1
ml/vial). Between 20 μ g and 40 μ g of β -gal plasmid
DNA was encapsulated in each glass vial. The glass
vials were left in the refrigerator for four days
to evaporate the methylene chloride and then
30 lyophilized.

Implantation of DNA/PLA pellets

Each sample was first sterilized with ethanol
for 5 min and then washed with PBS-
penicillin/streptomycin for 5 min. Surgery was
35 done on Sprague Dawley rats. Linear DNA was
implanted into the left leg and supercoiled DNA
implanted into the right. Implants were inserted
into incised muscle - either in the vastus or the

hamstring. The muscle was sutured back together and then the skin was sutured closed. Rats were sacrificed for analysis at two weeks.

Results

5 Rats were perfused with Phosphate Buffered Saline (PBS) with 2500 units of heparin followed by 3% paraformaldehyde and 0.2% glutaraldehyde in PBS. The tissue was post-fixed with 3% paraformaldehyde followed by 15% sucrose/PBS. Excised muscles were
10 cut with a cryostat and stained with X-gal.

Histology of the implant sites revealed a substantial inflammatory response around the film at two weeks and two months. The bulk of the β -gal positive staining was localized to this area with
15 few muscle cells exhibiting positive staining. The cells present around the implant probably consists of phagocytic cells, lymphocytes and fibroblasts. As expected, transfection was more efficient with supercoiled DNA.

20 Example 2: *In vitro* transfection with pRSV β -gal.

NIH3T3 fibroblasts were plated onto a 6 well tissue culture dish with 1 ml of D-MEM (10% Fetal calf serum with penicillin/streptomycin). 24 hours
25 after plating, the cells were transfected with pRSV β -gal control plasmids as per Promega Profection Mammalian Transfection system.

Plate 1: 10 μ l pRSV-Z (3.4 μ g) Calcium Phosphate Precipitated
30 Plate 2: 30 μ l pRSV-Z (10.2 μ g) Calcium Phosphate Precipitated
Plate 3: 10 μ l pRSV-Z (3.4 μ g) Naked DNA
Plate 4: 30 μ l pRSV-Z (10.2 μ g) Naked DNA
Plate 5: DNA/PLA
35 Plate 6: Control

Plate 5 with the PLA pellet was placed into the well with 4 ml of media to counter the effect of the decrease in pH. After 24 hours, the DNA/PLA pellet was removed and the media left unchanged.

At 48 hours, the cells were fixed and stained with X-Gal (1 ml/plate) overnight.

Results

The efficiency of transfection was very low.

- 5 All plates except the control well had a handful of blue staining cells. There was no observable differences in the number of blue cells among the 5 plates. It was interesting to note that the plate with the DNA/PLA had similar levels of staining as
10 the other plates even after the fact that half the cells had died and detached due to the PLA degradation.

Example 3: Duration of Expression with pSV β -gal DNA Encapsulated Into PLA Blends.

In vitro release of plasmid DNA

pSV β -gal was amplified in HB101 and purified with Qiagen's Mega Prep. 500 μ l of plasmid in Tris-EDTA buffer (67.5 μ g) was lyophilized and
20 resuspended into 100 μ l of sterile dH₂O and incorporated into PLA. 0.05 g PLA (2K) and 0.05 g (300K) was dissolved in 1 ml of methylene chloride and 1 drop of SpanTM 85. After the polymer was in solution, 100 μ l of plasmid (67.5 μ g) was added to
25 the mixture and vortexed for 15 sec. The resulting film was left in a refrigerator overnight and subsequently lyophilized overnight.

This film was incubated with 1.0 ml of TE buffer at 37°C and sample supernatants tested at 24
30 hours and at 4 days for the presence of released DNA. DNA was assayed by agarose gel electrophoresis on the supernatants.

The results based on the gel of the supernatant after 24 hours of incubation show that
35 a substantial amount of plasmid was released. After 4 days, the results indicate that there was a first phase of release due to the diffusion of plasmid molecules which are close to the surface of

the device followed by a slower release at 4 days due to the low degradation rate of the polymer which was too low to be measured.

In vivo transfection levels

5 3 mg PLA (2K) and 1 mg PLA (100K) were dissolved in methylene chloride (0.25 ml). 1 drop of Span™ 85 and 20 μ l of plasmid (20 μ g) was added to the solution and homogenized for 1 minute. This solution was air dried in a glass vial for 3 hours
 10 in a sterile hood. The brittle film was ground into fine granules and pressed into a pellet form. Three of these DNA containing pellets were made as well as three control pellets without DNA. All pellets were lyophilized overnight to extract
 15 residual solvents.

Three rats received DNA/PLA in their left hamstring and control/PLA in their right hamstring. Pellets were inserted into incised hamstrings and the muscles closed with 6-0 Vicryl. Three rats
 20 received an injection of pSV β -gal plasmids (20 μ g in 100 μ l of TE buffer) over a minute long period in their left leg and 100 μ l of plain TE buffer in their right leg as controls. The site of injection was marked with suture.

	<u>Rat ID</u>	<u>Left</u>	<u>Right</u>	<u>Implant Duration</u>
	R112	DNA/PLA	Control/DNA	1 week
	R110	DNA/PLA	Control/DNA	5 weeks
	R111	DNA/PLA	Control/DNA	10 weeks
30	R115	DNA/buffer	Control/buffer	1 week
	R114	DNA/buffer	Control/buffer	5 weeks

Rats were perfused with PBS/heparin, followed by 4% paraformaldehyde, and post-fixed in 4% paraformaldehyde followed by 15% and 25% sucrose/PBS. Excised muscles were cut with a
 35 cryostat and stained with X-Gal.

Results

In vitro release studies indicate that plasmid DNA can be incorporated into polymers without

degradation through manufacturing processes and released in functional form for possible uptake by surrounding cells.

In vivo studies reveal that with a 20 μ g loading of DNA into the polymer, there is substantial transfection of inflammatory cells at 1 and 5 weeks as confirmed by X-gal staining and immunoblotting. At 10 weeks, there was no difference in staining intensity between the control PLA and DNA/PLA. This is believed to be due to the result of the low loading (20 μ g) of the polymer such that after one week the release rate was below half maximal levels. Investigators using direct injection use doses in the 100 μ g range to see their effects. A higher initial loading, which will lead to continued release of higher amounts of DNA from polymers, should prolong transfection durations. Rats injected with 20 μ g of DNA in solution showed no transfection at 1 and 5 weeks.

20 **Example 4: Comparison of Plasmid DNA Release From Biodegradable and Non-degrading Polymers.**

Release of plasmids from biodegradable and non-degradable polymer was compared to test the feasibility of targeting either inflammatory cells or tissue specific cells by selection of polymer material. Plasmid DNA was incorporated into a non-degradable elastomer, ethylene vinyl acetate copolymer (EVAc) and implanted into the same site in different animals as PLA/PCL implants. EVAc is a very biocompatible polymer which can be manufactured into a microporous structure through which DNA can diffuse into the surrounding tissue.

Encapsulation of pRSV β -gal into Polymers.

35 pRSV β -gal in HB101 was purchased from the ATCC (American Type Culture Collection, Rockville, MD). The plasmids were grown and purified with Promega's Maxi Prep. 1 ml of a 0.1% solution of

ELVAX40 (Dupont) in methylene chloride was vortexed with 645.2 μ l of pRSV β -gal (200 μ g), frozen in liquid nitrogen and lyophilized. The resulting mixture was extruded at 55°C into a rod shaped 5 form.

PLA (2K) and PCL (112K) were dissolved in methylene chloride in a 3:1 ratio and 80 mg of the polymer vortexed with 322.6 μ l of pRSV β -gal (100 μ g). The mixture was left in the refrigerator for 10 2 days and lyophilized.

Implantation of the Polymers.

The EVAc/DNA and PLA/DNA were implanted into rat hamstrings along with their control on opposite sides and sacrificed at 2 weeks.

15 Results.

Histological staining with X-gal reveals positive staining of muscle cells as well as inflammatory cells in close proximity to the EVAc polymeric implant at two weeks post-implantation.

20 In comparison, the PLA/PCL implant reveals positive staining of mostly inflammatory cells only, in accordance with the earlier data regarding biodegradable polymers.

Thus the selection of a biodegradable or non-degradable polymer implant can be used to target 25 delivery to inflammatory cells or tissue cells (for example, muscle). Comparison of PLA/PCL and the EVAc implants illustrates the different transfected cell populations. Specifically, the PLA/PCL 30 implant results in almost exclusive transfection of inflammatory cells while the EVAc implant results in a large number of transfected muscle cells.

Example 5: Stability of plasmids during manufacturing processes.

35 Plasmid DNA is known to be sensitive to high temperatures, physical manipulations and other such factors. Because fabrication techniques require different manipulation of plasmids, methods must be

selected to avoid substantial degradation of plasmids. The following studies were conducted to determine the effect of exposure of plasmids to heating, sonication and solvents such as methylene chloride, as analyzed by agarose gel electrophoresis for changes in topology and/or degradation.

Effect of heating

Lyophilized plasmids were added to dry polymer (PCL) and heated up to 85°C for 5 minutes and swirled into a 85°C corn oil bath as per hot melt microsphere preparation protocols. Electrophoresis of extracted plasmids indicate that the majority of the loaded plasmids had lost their supercoiled topology and degraded into a linear form.

Effect of sonication

A mixture of polymer (PCL) and plasmid solution was sonicated for 5 seconds. Electrophoresis of the extracted plasmids show that there is some degradation from sonication. Thus, sonication of plasmid/polymer solutions for dispersion in the polymer matrix can result in substantial degradation of plasmids.

Effect of methylene chloride exposure

Lyophilized plasmids and plasmids in solution were exposed to methylene chloride and vortexed together for 30 seconds. Agarose gel electrophoresis of the extracted plasmids show that there are no detectable degradation due to methylene chloride exposure.

Results

These experiments illustrate that plasmids are sensitive to some of the physical manipulations required by various fabrication techniques and can result in the incorporation of linear, nicked or degraded plasmids. *In vitro* and *in vivo* transfection efficiency is highest with supercoiled

DNA. Thus, manufacturing methods should be selected or optimized to preserve the supercoiled topology of the DNA within the polymer matrix.

Example 6: *In vitro* release assay.

5 *In vitro* evaluation of plasmid releasing polymer systems was performed by incubating the devices in buffers such as Tris-EDTA and periodically replacing the supernatant with fresh buffer. The incubation buffer was analyzed for
10 plasmids with agarose gel electrophoresis to assess plasmid topology. In addition, the plasmids present in the release buffers were precipitated with the Promega™ Profection Calcium Phosphate Mammalian Transfection System and transfected into
15 C2C12 murine muscle cells to assay for bioactivity.

Materials tested

- 1) Ethylene vinyl acetate (EVAc) ELVAX®40
- 2) Polylactic acid (PLA) Mw 2k / polycaprolactone (PCL) Mw 112k-3:1 blend
- 20 3) Polylactic acid (PLA) Mw 2k / polycaprolactone (PCL) Mw 75k - 2:1 blend
- 4) Polycaprolactone (PCL) Mw 75k
- 5) Polylactic acid (PLA) Mw 2K / polylactic acid (PLA) Mw 300k - 2:1 blend
- 25 6) Polylactic acid (PLA) Mw 2k / polylactic acid (PLA) Mw 100k - 2:1 blend
- 7) Fatty acid dimer (FAD) / sebacic acid (SA:FA, 50:50)

30 The fabrication methods, materials, and results are summarized in Table 1.

-32-

Table 1: In Vitro Release of Plasmids

POLYMER	FORM	FABRICATION METHOD	IN VITRO RELEASE RESULTS
EVAC	films	Heat pressed (500C)	Undegraded
	rods	Heat extrusion (550C)	Undegraded
EVAC	pellets	Pressed	Partial Degradation
	microspheres	Solvent evaporation	Unavailable
PLA/PCL (112k)	films	Solvent cast	Undegraded
	microspheres	Solvent removal	Undegraded, detectable at one week
PLA/PCL (112k)	films	Hot melt (850C)	Linearized
	microspheres	Solvent cast	Unavailable
PLA (75k)	pellets	Pressed	Partial Degradation
	films	Heat extrusion (500C)	Degraded
PLA/PCL (75k)	rods	Heat extrusion (500C)	Undegraded
	microspheres	Solvent precipitation	Partial Degradation
PCL	microspheres	Hot melt (850C)	Linearized
PLA (2k) / PLA (300k)	films	Solvent cast	Unavailable
PLA (2k) / PLA (100k)	pellets	Pressed	Partial Degradation
FAD/SA	rods	Heat extrusion (500C)	Degraded
FASA (20:80)	microspheres	Solvent precipitation	Partial Degradation

The plasmids were evaluated by agarose gel electrophoresis of the plasmids in the release buffer. The results showed degraded and intact supercoiled plasmids in the samples of pRSV/βgal loaded PLA/PCL (112k), pRSV/βgal loaded PLA/PCL (112k), and pRSV/βgal loaded PLA/PCL (112k).

5 Agarose gel electrophoresis show undegraded plasmids in the release buffer, as reflected by clean bands, in pRSV/βgal loaded EVAc, pRSV/βgal 10 loaded EVAc. and pRSV/βgal loaded EVAc.

This provides further evidence that the effect of processing conditions on the plasmids can be controlled by selection of the polymer and processing conditions.

15 The plasmids were also evaluated for biological activity. Supernatents containing the released plasmids were precipitated with calcium phosphate and transfected into C2C12 muscle cells according to Promega™ Profection protocols. Cells 20 were assayed for β-galactosidase activity 48 hours post-transfection with X-gal histochemistry. The results obtained with positive control stock pSV/βgal (10 µg) CaP₄ transfection of C2C12 cells were compared with the results obtained with CaP₄. 25 transfection with the DNA released over a period of four weeks into the supernatant from EVAc-pSV/βgal rods.

The *in vitro* transfection efficiency, as measured by the number of β-gal positive cells, of 30 released plasmids were lower than that seen with stock plasmid DNA. One explanation for this difference could be due to the fact that stock plasmids are 95% monomeric supercoiled plasmids while the plasmids released from the polymers have 35 open circular or multimeric supercoiled topology.

However, as demonstrated above, the release occurs over a longer period of time than can be obtained with stock plasmid DNA.

5 **Example 7: Microsphere delivery in rat aorta/adventitial layer for plasmid release targeting vascular smooth muscle and endothelium.**

10 The following study was conducted to demonstrate that the microspheres encapsulating plasmids could be used to target smooth muscle and endothelium for release of plasmids directly to these cells.

Methods and Materials:

15 A segment of the aorta/vena cava approximately 2 centimeters in length was isolated. A small hole in the adventitia was cut for insertion of a 27 gauge catheter. A blunt 25 gauge needle was used to separate the adventitia from the medial layer. A 27 gauge catheter was inserted into the space and 20 the microspheres were injected.

10 mg of 100 μ m sieved PLGA/PCL microspheres suspended in a sodium hyaluronate solution were injected into the aorta.

25 Cryosections were then made of the microspheres in the rat aorta. The results demonstrate the feasibility of the method and that the volume should be minimized to the extent possible, preferably fifty microliters.

30 **Example 8: Release from pSV/ β gal PLA:PCL(2:1) microspheres.**

The following study was to demonstrate that release rate is a function of degradation rate of the polymer used for release.

35 Microspheres were made by solvent removal from polylactic acid (PLA) (Mw 2k) and polycaprolactone (PCL) (Mw 75k) (2:1). Samples contain 1% loading of pSV/ β gal dispersed dry or wet.

The results show that release was undetectable after one week, probably due to the slow

degradation of the polymer. Faster release can be obtained using a faster degrading polymer such as poly(FA:SA) fumaric co-sebacic acid. Another explanation could be due to the fact that with one 5 of the polymer blends used, the microspheres formed were double walled. Thus the outer layer could be slowing the release of the DNA contained in the inner core.

10 **Example 9: Release of Liposome/DNA Complexes with Ethylene Vinyl Acetate polymers.**

Liposome/DNA complexes have been used for both 15 *in vitro* and *in vivo* transfection of mammalian cells. For *in vitro* applications, various formulations such as LIPOFECTIN™ (GIBCO BRL) and TRANSFECTAM™ (Promega) have been used to enhance 20 cellular uptake of plasmid DNA. Although liposome/DNA complexes have high transfection efficiencies when used in tissue culture, direct application of liposome/DNA for *in vivo* 25 applications is somewhat limited. Limitations on the use of liposome/DNA complexes *in vivo* stem from their fast degradation, toxicity at high doses and inability to transfect certain cell types. In the case of skeletal muscle, direct injection of 30 plasmid DNA is much more efficient than liposome delivery. Nonetheless, in certain tissues such as the brain, liposome/DNA results in much higher expression levels compared to direct injection.

35 This study was done to assess the feasibility of releasing liposome/DNA complexes from polymer systems. In light of the fact that lipids are soluble in organic solvents, preserving the liposome/DNA complex in intact form within the polymer matrix was the primary goal.

Materials:

200 μ l of pSV/Bgal (1 μ g/ μ l)

200 μ l Lipofectin™

100 μ l dH2O

5 1 ml EVAc/Methylene Chloride (5%
solution)

Method:

The plasmid DNA and lipid solutions were vortexed briefly and allowed to form complexes for 10 min. This solution was vortexed for 10 seconds with the polymer solution, frozen in liquid nitrogen and lyophilized overnight. The resulting matrix was either used with or without heat extrusion at 50°C.

15 To assay the bioactivity of the liposome/DNA complexes, the extruded rod and unextruded matrix (10 mg each) was incubated with C2C12 myoblasts for 48 hours. The cells were subsequently fixed and stained for β -galactosidase activity by X-gal

20 histochemistry.

The results show that incubation with the unextruded matrix resulted in positive transfection while incubation with the extruded matrix resulted in no transfection. Subsequent experiments with 25 the unextruded matrix show that liposome/DNA complexes are released for up to one week in vitro.

Modifications and variations of the method and compositions of the present invention will be obvious to those skilled in the art from the 30 foregoing detailed description. Such modifications and variations are intended to come within the scope of the following claims.

We claim:

1. A delivery system for a gene into a cell comprising
a biocompatible polymeric device and
a gene under the control of a promoter which
is capable of being expressed in a mammalian cell,
wherein the gene is entrapped in the polymer
and released into the surrounding cells when the
polymeric device is implanted into tissue.
2. The system of claim 1 wherein the
polymeric device is selected from the group
consisting of microparticles, microspheres,
microcapsules, stents, coatings, implants, slabs,
films, and gels.
3. The system of claim 1 wherein the
polymeric device is formed of a biodegradable
polymer.
4. The system of claim 3 wherein the polymer
is selected from the group consisting of synthetic
polymers, polysaccharides, proteins, and
combinations thereof.
5. The system of claim 1 further comprising a
component selected from the group consisting of
compounds inhibiting inflammation due to the
polymer device and compounds increasing
inflammation due to the polymeric device.
6. The system of claim 1 wherein the gene
includes a promoter and a transcription termination
signal.
7. The system of claim 1 further comprising
compounds selected from the group consisting of
antiinflammatories, inhibitors of capsule
formation, and inhibitors of cytokines.
8. The system of claim 1 wherein the polymer
is bioadhesive.
9. The system of claim 1 comprising between
0.01 and 90% by weight gene.

10. A method for delivery of a gene into a cell *in vivo* comprising
 entrapping a gene under the control of a promoter which is capable of being expressed in a mammalian cell in a biocompatible polymeric device which releases the gene following implantation of the polymer.
11. The method of claim 10 wherein the polymeric device is selected from the group consisting of microparticles, microspheres, microcapsules, stents, coatings, implants, slabs, films, and gels.
12. The method of claim 10 wherein the polymeric device is formed of a biodegradable polymer.
13. The method of claim 12 wherein the polymer is selected from the group consisting of synthetic polymers, polysaccharides, proteins, and combinations thereof.
14. The method of claim 10 further comprising a component selected from the group consisting of compounds inhibiting inflammation due to the polymer device and compounds increasing inflammation due to the polymeric device.
15. The method of claim 10 wherein the gene includes a promoter, a transcription termination signal, and the promoter is tissue specific.
16. The method of claim 10 further comprising administering with the device antiinflammatories, inhibitors of capsule formation, and inhibitors of cytokines.
17. The method of claim 10 for targeting release of the gene into inflammatory cells comprising selecting a biodegradable polymer as the polymer forming the device.

18. The method of claim 10 for targeting release of the gene into inflammatory cells comprising selecting a polymer which elicits an inflammatory reaction as the polymer forming the device.

19. The method of claim 10 for targeting release of the gene into tissue specific cells comprising selecting a non-biodegradable polymer as the polymer forming the device.

20. The method of claim 10 for targeting release of the gene into tissue specific cells comprising selecting a biocompatible polymer as the polymer forming the device.

21. The method of claim 10 further comprising selecting a bioadhesive polymer as the polymer forming the implant.

22. The method of claim 10 further comprising loading between 0.01 and 90% by weight gene into the polymer.

23. A method for delivery of nucleic acid molecules comprising incorporating the nucleic acid molecules in combination with liposomes into a matrix formed of a biocompatible polymer which releases the nucleic acid molecules and liposomes following implantation of the polymeric matrix.

22. The method of claim 23 wherein the polymeric device is selected from the group consisting of microparticles, microspheres, microcapsules, stents, coatings, implants, slabs, films, and gels.

23. The method of claim 21 wherein the polymeric device is formed of a biodegradable polymer and the liposomes are formed of cationic lipids.

24. The method of claim 21 wherein the nucleic acid molecules are selected from the group consisting of genes under the control of a promoter, antisense, ribozymes and ribozyme targeting molecules, and probes.

25. A composition for delivery of nucleic acid molecules comprising nucleic acid molecules in combination with liposomes incorporated into a matrix formed of a biocompatible polymer which releases the nucleic acid molecules and liposomes following implantation of the polymeric matrix.

26. The composition of claim 25 wherein the polymeric device is selected from the group consisting of microparticles, microspheres, microcapsules, stents, coatings, implants, slabs, films, and gels.

27. The composition of claim 25 wherein the polymeric device is formed of a biodegradable polymer and the liposomes are formed of cationic lipids.

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<p>(51) International Patent Classification 6 : A61K 9/16, 9/50, 48/00</p>		A3	<p>(11) International Publication Number: WO 95/24929</p> <p>(43) International Publication Date: 21 September 1995 (21.09.95)</p>
<p>(21) International Application Number: PCT/US95/03307</p> <p>(22) International Filing Date: 15 March 1995 (15.03.95)</p> <p>(30) Priority Data: 213,668 15 March 1994 (15.03.94) US</p> <p>(71) Applicant: BROWN UNIVERSITY RESEARCH FOUNDATION [US/US]; Box 1949, Providence, RI 02912 (US).</p> <p>(72) Inventors: MATHIOWITZ, Edith; 184 Rawson Road, Brookline, MA 02146 (US). JONG, Yong, Shik; Hankang Minyoung Apartment L-301, Dongbu-Ichon Dong, Seoul (KR). BOEKELHEIDE, Kim; 154 Pine Hill Road, Wakefield, RI 02879 (US).</p> <p>(74) Agent: PABST, Patrea, L.; Arnall Golden & Gregory, 2800 One Atlantic Center, 1201 West Peachtree Street, Atlanta, GA 30309-3450 (US).</p>		<p>(81) Designated States: AU, CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).</p> <p>Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i></p> <p>(88) Date of publication of the international search report: 28 December 1995 (28.12.95)</p>	
<p>(54) Title: POLYMERIC GENE DELIVERY SYSTEM</p> <p>(57) Abstract</p> <p>A means for obtaining efficient introduction of exogenous genes into a patient, with long term expression of the gene, is disclosed. The gene, under control of an appropriate promoter for expression in a particular cell type, is encapsulated or dispersed with a biocompatible, preferably biodegradable polymeric matrix, where the gene is able to diffuse out of the matrix over an extended period of time, for example, a period of three to twelve months or longer. The matrix is preferably in the form of a microparticle such as a microsphere (where the gene is dispersed throughout a solid polymeric matrix) or microcapsule (gene is stored in the core of a polymeric shell), a film, an implant, or a coating on a device such as a stent. The size and composition of the polymeric device is selected to result in favorable release kinetics in tissue. The size is also selected according to the method of delivery which is to be used, typically injection or administration of a suspension by aerosol into the nasal and/or pulmonary areas. The matrix composition can be selected to not only have favorable degradation rates, but to be formed of a material which is bioadhesive, to further increase the effectiveness of transfer when administered to a mucosal surface.</p>			

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	GB	United Kingdom	MR	Mauritania
AU	Australia	GE	Georgia	MW	Malawi
BB	Barbados	GN	Guinea	NE	Niger
BE	Belgium	GR	Greece	NL	Netherlands
BF	Burkina Faso	HU	Hungary	NO	Norway
BG	Bulgaria	IE	Ireland	NZ	New Zealand
BJ	Benin	IT	Italy	PL	Poland
BR	Brazil	JP	Japan	PT	Portugal
BY	Belarus	KE	Kenya	RO	Romania
CA	Canada	KG	Kyrgyzstan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo	KR	Republic of Korea	SE	Sweden
CH	Switzerland	KZ	Kazakhstan	SI	Slovenia
CI	Côte d'Ivoire	LI	Liechtenstein	SK	Slovakia
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CN	China	LU	Luxembourg	TD	Chad
CS	Czechoslovakia	LV	Latvia	TG	Togo
CZ	Czech Republic	MC	Monaco	TJ	Tajikistan
DE	Germany	MD	Republic of Moldova	TT	Trinidad and Tobago
DK	Denmark	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	US	United States of America
FI	Finland	MN	Mongolia	UZ	Uzbekistan
FR	France			VN	Viet Nam
GA	Gabon				

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 95/03307A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K9/16 A61K9/50 A61K48/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, X	WO,A,94 23738 (MEDISORB TECHNOLOGIES INTERNAT) 27 October 1994 see claims 1-8 ---	1-27
X	EP,A,0 579 347 (FERRING ARZNEIMITTEL GMBH) 19 January 1994 see column 3, line 5 - line 47 ---	1-27
Y	US,A,5 100 669 (HYON SUONG-HYU ET AL) 31 March 1992 see column 3, line 25 - column 4, line 68; claims 1-6 --- -/-	1-27

 Further documents are listed in the continuation of box C. Patent family members are listed in annex.

* Special categories of cited documents :

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *B* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

- *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- *&* document member of the same patent family

4 Date of the actual completion of the international search

11 October 1995

Date of mailing of the international search report

24.11.95

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentiaan 2
NL - 2280 HV Rijswijk
Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl,
Fax (+ 31-70) 340-3016

Authorized officer

Foerster, W

INTERNATIONAL SEARCH REPORT

Int. J. Appl. Pat. No.
PCT/US 95/03307

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	PROC. NATL. ACAD. SCI. USA, vol.90, 3 pages 11307 - 11311 G. NABEL ET AL. 'Direct gene transfer with DNA-liposome complexes in melanoma: Expression, biologic activity, and lack of toxicity in humans' cited in the application see the whole document ---	1-27
A	WO,A,93 25221 (ALKERMES CONTROLLED THERAPEUTI) 23 December 1993 see the whole document ---	1-27
A	WO,A,93 08850 (MASSACHUSETTS INST TECHNOLOGY ;CHILDRENS MEDICAL CENTER (US)) 13 May 1993 see the whole document ---	1-27
A	FASEB JOURNAL FOR EXPERIMENTAL BIOLOGY, vol.7, no.11, , BETHESDA, MD US pages 1061 - 1069 I.T. TAI ET AL. 'Microencapsulation of recombinant cells: a new delivery system for gene therapy' see the whole document -----	1-27

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int. Search Application No

PCT/US 95/03307

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9423738	27-10-94	AU-B-	6707194	08-11-94
EP-A-0579347	19-01-94	DE-C-	4223169	25-11-93
US-A-5100669	31-03-92	JP-A- EP-A, B	1216918 0330180	30-08-89 30-08-89
WO-A-9325221	23-12-93	AU-B- CA-A- EP-A- JP-T-	4630893 2136434 0644771 7507806	04-01-94 23-12-93 29-03-95 31-08-95
WO-A-9308850	13-05-93	CA-A- EP-A- JP-T-	2121040 0610423 7508663	13-05-93 17-08-94 28-09-95